PCT WORLD INTELLECTUAL PROPER
International Bure

INTERNATIONAL APPLICATION PUBLISHED UNDER T WO 9605850A1

(51) International Patent Classification 6 : A61K 38/00 | A1

(11) International Publication Number: WO 96/05850

(43) International Publication Date: 29 February 1996 (29.02.96)

(21) International Application Number: PCT/US95/10457

(22) International Filing Date: 17 August 1995 (17.08.95)

(30) Priority Data:
08/292,413 18 August 1994 (18.08.94) US

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(81) Designated States: AU, CA, CN, FI, JP, KR, NO, NZ, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CYTOKINE REGULATION OF CELLULAR SENESCENCE

(57) Abstract

The present invention provides a novel method of decreasing cellular senescence comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal. Also provided are pharmaceutical compositions for use in the methods of the present invention.

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CYTOKINE REGULATION OF CELLULAR SENESCENCE

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of immunology and protein chemistry. More specifically, the present invention relates to the novel use of a cytokine to inhibit cellular senescence.

Description of the Related Art

Loss of proliferative potential of normal human cells in culture, initially described in normal human diploid fibroblasts, is widely used as a reliable *in vitro* model of aging. This model of cellular senescence has now been reproduced with a variety of differentiated cell types including vascular endothelial cells, lymphocytes, and adrenocortical cells.

Why cells stop dividing after certain number of population doublings is unknown. Cell fusion experiments have suggested that senescence is a dominant phenotype and that immortal cell lines result from an inactivation of key cell senescence related genes. The latter have been assigned to four distinct complementation groups. Cellular senescence is accompanied by several changes including: (1) a decrease of cell cycle related enzyme ornithine decarboxylase and thymidine kinase activities; (2) a decrease in protein synthesis and degradation; (3) a decrease in the length of telomeres and of 5-methylcytosine content in DNA; and (4) the presence of higher frequency of nuclear and chromosomal aberrations. Phenotypically, senescent cells have been shown to be larger and grow to a lower saturation density as compared to young counterparts.

The age-dependent loss of cellular proliferation has been shown to be due to the inability to phosphorylate retinoblastoma gene product, failure to express cell cycle-dependent genes such as c-fos, cdc 2 or cyclins and acquisitions of factors such as senescence-associated genes (SAG), terminin and sdi (senescence derived inhibitor) that prevent

proliferation. Thus, it is possibl that cellular senescence is not due to down-regulation of proliferative factors but is instead due to up-regulation of antiproliferative factors. During the last decade, several different novel proliferative and antiproliferative growth factors have been identified which control the replicative potential of cells. Most of these factors, also referred as cytokines, are polypeptide hormones which differentially regulate cell growth in a cell type specific manner. Among the cytokines, it has been shown that basic fibroblast growth factor, tumor necrosis factor and interleukin-1 could induce the proliferation of young normal human diploid fibroblasts, an *in vitro* model commonly used for aging. Recently, these cytokines were shown to induce proliferation of young cells and have no effect on senescent human diploid fibroblasts. How various growth factors modulate the limited *in vitro* long-term life span of these cells, is not known.

The prior art is deficient in the lack of effective means of inhibiting cellular senescence. The prior art is also deficient in the lack of varied and diverse pharmacological tools to treat non-malignant hyperproliferative diseases. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention illustrates the effect of ten different cytokines on the age-dependent proliferation of normal human diploid fibroblasts (HDF) cells in culture. As disclosed herein, certain cytokines can increase the life span of fibroblasts and postpone the senescence process. As also disclosed herein, certain other cytokines are very effective in significantly decreasing fibroblast proliferation.

In one embodiment of the present invention, there is provided a method of decreasing cellular senescence comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal.

In another embodiment of the present invention, there is provided a pharmaceutical composition for the treatment of cellular senescence comprising a pharmacologically effective dose

of basic fibroblast growth factor and a pharmaceutically acceptable carrier.

In yet another embodiment of the present invention, there is provided a method of a method of increasing endogenous levels of insulin growth factor-1 (IGF-1) in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal.

In still yet another embodiment of the present invention, there is provided a method of decreasing endogenous levels of senescence inducing protein in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal.

In another embodiment of the present invention, there is provided a method of treating a pathophysiological state characterized by undesirable fibroblast proliferation, comprising the administration of a pharmacologically effective dose of a fibroblast-inhibiting cytokine to an animal having said state.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the effect of interferon-alpha on the life span of human diploid fibroblasts. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with interferon-alpha at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 2 shows the effect of interleukin-6 on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with interleukin-6 at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 3 shows the effect of interleukin-4 on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with interleukin-4 at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 4 shows the effect of leukemia inhibitory factor (LIF) on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with leukemia inhibitory factor at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 5 shows the effect of tumor necrosis factor (TNF) on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with tumor necrosis factor at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 6 shows the effect of transforming growth factor-beta on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with transforming growth factor-beta at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 7 shows the effect of interferon-gamma on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with interferon-gamma at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 8 shows the effect of interleukin1-beta on the life span of human diploid fibroblasts. 4 X10^4 cells (2 ml) in 24-well plates were incubated with interleukin1-beta at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 9 shows the effect of interferon-beta on the life span of human diploid fibroblasts. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with interferon-beta at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 10 shows the effect of basic fibroblast growth factor on the life span of human diploid fibroblasts. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with interferon-beta at 37°C for 7 days and then cells were trypsinized and viable cell number determined by trypan blue exclusion.

Figure 11 shows the effect of basic fibroblast growth factor on the replicative potential of human diploid fibroblasts at different population doublings. 4 $X10^3$ cells (0.2 ml) in 24-well plates were incubated with the cytokine at 37°C for 7 days, then trypsinized and viable cell number determined by trypan blue exclusion.

Figure 12 shows the effect of different concentrations of tumor necrosis factor (Figure 12A), interleukin-1 (Figure 12B) and basic fibroblast growth factor (Figure 12C) on young and senescent fibroblast cells. 8 $X10^3$ cells (0.2 ml) in 96-well plates were incubated with the cytokine at 37°C for 5 days and then thymidine uptake was determined during last 24 hours. All determinations were made in triplicate.

Figure 13 shows the effect of human interferon-alpha on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 14 shows the effect of human interleukin-4 on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 8 and 13 weeks. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and th n cells were photographed at different population doublings.

Figure 15 shows the effect of human interleukin-6 on the morphology of human diploid fibroblasts at different

population doublings, i.e., 4, 13, 14 and 18 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 16 shows the effect of human leukemia inhibitory factor on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 17 shows the effect of human interleukin-1B on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 8 and 9 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 18 shows the effect of human tumor necrosis factor on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 6, 8 and 9 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 19 shows the effect of human interferon-γ on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 20 shows the effect of human interferon-β on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. 4 X10^4 cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population d ublings.

Figure 21 shows the effect of human transforming growth factor-β on the morphology f human diploid fibroblasts at different population doublings, i.e., 4 and 6 weeks. 4 X10^4

cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 22 shows the effect of human basic fibroblast growth factor on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13, 14 and 17 weeks. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

Figure 23 shows the effect of aging on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 17 weeks. 4 $X10^4$ cells (2 ml) in 24-well plates were incubated with different cytokine at 37°C for 7 days and then cells were photographed at different population doublings.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of decreasing cellular senescence comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal. Generally, the methods of the present invention are equally advantageous and of desirable use in treating various animals, including mammals. Most preferably, the methods of the present invention would be most useful in a human.

The present invention is also directed to a method of increasing endogenous levels of insulin growth factor-1 (IGF-1) in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal.

In a separate embodiment, the present invention pertains to a method of decreasing endogenous levels of senescence inducing proteins in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal. Most preferably, the senescence inducing protein is sdi or senescence derived inhibitor.

A pharmaceutical composition, comprising basic fibroblast growth factor and a pharmaceutically acc ptable carri r is also provided. The pharmaceutical compositions of the

pr sent invention are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science*, 249:1527-1533 (1990). Methods for preparing administrable compounds will be known or apparent to those skilled in the art and are described in more detail, for example, in Remington's *Pharmaceutical Science*, 17th ed., Mack Publishing Company, Easton, PA (1988). A person having ordinary skill in this art would readily recognize the most appropriate route of administration and dosages for fibroblast growth factor. Preferably, basic fibroblast growth factor is administered in a daily amount of from about 1 mg/kg to about 100 mg/kg.

Accordingly, the present invention provides a pharmaceutical composition for the treatment of cellular senescence comprising a pharmacologically effective dose of basic fibroblast growth factor and a pharmaceutically acceptable carrier.

Administration of basic fibroblast growth factor in the methods of the present invention may be by topical, parenteral, oral, intranasal, intravenous, intramuscular, subcutaneous, or any other suitable means. The preferred method of administration for treatment of skin cell proliferative diseases is by topical application or subcutaneous injection.

The present invention also provides a method of treating a pathophysiological state characterized by undesirable fibroblast proliferation, comprising the administration of a pharmacologically effective dose of a fibroblast-inhibiting cytokine to an animal having said state. Representative examples of relevant pathophysiological state include psoriasis, arthritis, restenosis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, scleroderma and hemangioma. As is discussed more fully below, the fibroblast-inhibiting cytokine useful in treating pathophysiological states characterized by uncontrollable and/or undesirable fibroblast proliferation include tumor necrosis factor, transforming growth factor-β, interferon-β, interferon-gamma and interleukin-1β. As shown in the present invention, these specific cytokines may be useful

in r gulating a wide vari ty of conditions where control of fibroblast proliferation is b neficial or advantag ous. A person having ordinary skill in this art would readily recognize or be able to determine without undue experimentation, the most appropriate doses of tumor necrosis factor, transforming growth factor-β, interferon-β, interferon-gamma and interleukin-1β as these cytokines have become useful for various other medical conditions.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Materials

Bacteria-derived recombinant human TNF, IL-1b, interferons α and γ (IFN-α, IFN-γ) and transforming growth factor-β (TGF-β) were provided by Genentech Inc., South San Francisco, CA; human fibroblast interferon (IFN-β) was obtained from Toray Company, Kamakura, Japan. IL-4 was a gift of Schering-Plough Co., NJ. IL-6 was a gift of Sandoz Pharmaceutical Corp., East Hanover, NJ. Leukemia inhibitory factor (LIF) was a gift of Amgen, Thousand Oaks, CA. Basic fibroblast growth factor (b-FGF) was provided by Chiron Corp., Emeryville, CA. All cytokines were highly purified recombinant human proteins. Other chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Dulbeco's modified Eagle's medium (DMEM) was obtained from Whittaker MA Bioproducts (Walkersville, MD). RPMI 1640 medium, fetal bovine serum and gentamicin were obtained from GIBCO, Grand Island, NY. 12-well plates were obtained from Becton Dickinson and Co. (Oxnard, CA; Lincoln Park, NJ). Primary human foreskin fibroblasts at early passages were supplied by Dr. Olivia Smith of Baylor College of Medicine, Houston, TX.

EXAMPLE 2

Cells

Human foreskin fibroblasts cultures w re maintained in continuous xponential growth by weekly passage. Cells were routin ly grown in RPMI 1640 medium supplemented with glutamin

(2 mM), gentamicin (50 μg/ml), and fetal bovine serum (10%) in a humidified incubator in 5% CO_2 in air.

EXAMPLE 3

Fibroblast Proliferation Assays

Young human foreskin fibroblasts at seeding population doublings of 22.6 (4 x 10^4 cells) were plated into 12 well plates in 2 ml RPMI 1640 media containing 10% FBS. The next day, the media was removed and fresh media containing different cytokines (20 ng/ml) was added into the wells. After 7 days, the confluent cells were trypsinized, counted by trypan blue exclusion method and the population doublings calculated as described below:

$N_H / N_I = 2^X$

(N_H = cell harvest number, N_I = cell inoculum number, and X = the number of population doublings). The increase in population doubling level (PDL) was added to the previous population doubling level to arrive at the current cumulative population doubling level. Each week 0.04 x 10^6 cells in 12-well plates were cultured in the presence of fresh cytokine-containing media. The cells were examined for mycoplasma every six months by DAPI staining method. DAPI or 4-6-diamine-2-phenyl-indole dihydrochloride is a fluorescent dye which selectively binds to DNA and strongly forms fluorescent DNA-DAPI complexes. Mycoplasmas present in the cytoplasm appear as strongly fluorescent areas. The cytochemical detection of mycoplasma with DAPI was performed according to the manufacturer's, Boehringer Mannheim, suggestions.

EXAMPLE 4

Determination of Interleukin-6

Human diploid fibroblasts (0.04 x 10^6 cells in 2 ml) were incubated with different cytokines (20 ng/ml) for 7 days and then the supernatants were collected. The presence of IL-6 was determined by using the IL-6 dependent murine B cell hybridoma, B-9 cell line described by Schmidt, et al., *J. Immunol.*, 128:2177 (1982). Briefly, 2 x 10^3 cells were incubated in 0.2 ml of PRMI-1640 containing 10% FBS in 96-well plates with different concentrations of either IL-6 or IL-6 containing supernatants f r

96 hours. During the last 6 hours, cells were pulsed with 0.5 μCi of tritiated thymidine, harvested and cell associated radioactivity counted as described for cell proliferation assay. Fifty per cent of the maximum thymidine incorporation was defined as one unit which was obtained with one picogram of IL-6.

EXAMPLE 5

Effect of Cytokines on the Life Span of HDF

To illustrate the effect of different cytokines on the total life span of human diploid fibroblasts, cells were grown continuously in the presence various cytokines, subcultivated every week, cell number counted and same number replated, i.e., the number of cells that were plated in the last immediate passage. As shown in Figures 1-4, Interferon-α, Interleukin-4, Interleukin-6 and Leukemia Inhibitory Factor has no effect on the life span of human diploid fibroblasts as compared to the control, media alone. Normal human diploid fibroblasts are known to secrete Interleukin-6. However, the present invention shows that Interleukin-6 does not effect the long-term growth of human diploid fibroblasts.

The effect of cytokines, tumor necrosis factor, transforming growth factor-β, interferon-β, interferon-gamma and interleukin-1β, was to reduce the life span of human diploid fibroblasts as compared to the control (Figures 5-9), thus resulting in premature aging. Since normal human diploid fibroblasts are known to express Transforming Growth Factor-β, Interferon-β and Interleukin-1β, these cytokines can reduce the life span of the cells in an autocrine manner. The present invention suggests that antibodies to Transforming Growth Factor-β, Interferon-β and Interleukin-1β may enhance the life span of these cells.

Surprisingly, human TGF-β was found to be the most potent inhibitor of human diploid fibroblasts proliferation, since ming lung fibroblasts have been shown to proliferate in respons to this cytokine. The effects seen in the present inv ntion with tumor n crosis factor are unexpected because in short-term culture tumor necrosis factor enhances the proliferation of human diploid fibroblasts.

In contrast to all the other cytokines tested, basic-fibroblast growth factor was found to enhance the total life span of human diploid fibroblasts (Figure 10). As compared to the control, an approximately 60% increase in population doublings was observed when cells were grown in the presence of human diploid fibroblasts. No significant proliferation of untreated cells was observed after 9 weeks whereas basic fibroblast growth factor-treated cells (even after 14 weeks) had not developed a classical senescent morphology characterized by increase in cell size and sparse growth. When analyzed for replicative potential, basic fibroblast growth factor- treated cells have higher replicative potential as compared to the control cells at all passage levels (Figure 11). At week 11, the control cells reached complete senescence whereas basic fibroblast growth factor-treated cells had a weekly replicative potential of 3.6. Thus, the present invention clearly indicates that basic fibroblast growth factor has a highly significant effect on the life span of human diploid fibroblasts. An extracellular factor that increases programmed cellular senescence of fibroblasts has heretobefore never been described.

EXAMPLE 6

Continuous Presence of Cytokines is Needed

The need of human diploid fibroblasts to be exposed to cytokines was evaluated while cells were young or whether senescent cells can undergo proliferation when exposed to cytokines. As shown in Figure 3, tumor necrosis factor (Figure 3A), basic-fibroblast growth factor (Figure 3B) and IL-1β (Figure 3C) induce the proliferation of young human diploid fibroblasts. However, these cytokines had no effect on senescent human diploid fibroblasts. Thus, cytokines must be present at early stages in order to display their effects on long-term growth.

EXAMPLE 7

Cytokine Induced Morphological Alterations

It is known that young human diploid fibroblasts grow densely and are spindle-shaped. In contrast, th senescent human diploid fibroblasts exhibit flattish and sparse morphology and th y are bigg r in size than young cells. As the cells advanc

toward the senescent morphology, they first stop dividing and then there is a change in phenotype.

Figure 13 shows the effect of human interferon-alpha on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks.

Figure 14 shows the effect of human interleukin-4 on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 8 and 13 weeks.

Figure 15 shows the effect of human interleukin-6 on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13, 14 and 18 weeks.

Figure 16 shows the effect of human leukemia inhibitory factor on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks.

Figures 17-21 illustrate the effect of cytokines which decrease fibroblast proliferation. Figure 17 shows the effect of human interleukin-1B on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 8 and 9 week. Figure 18 shows the effect of human tumor necrosis factor on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 6, 8 and 9 weeks. Figure 19 shows the effect of human interferon-γ on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. Figure 20 shows the effect of human interferon-β on the morphology of human diploid fibroblasts at different population doublings, i.e., 4, 13 and 14 weeks. Figure 21 shows the effect of human transforming growth factor-β on the morphology of human diploid fibroblasts at different population doublings, i.e., 4 and 6 weeks.

As can be noted in Figure 22, the basic-fibroblast growth factor treated cells even at week 14 with a population doubling of 71.2 display the morphology of control cells at week 4 with population doubling of 32.5. Between week 14 and 17, the basic- fibroblast growth factor treated cells do not divid significantly but they acquire senescent phenotype. Thus, it is clear that basic- fibroblast growth factor treatment of human

diploid fibroblasts improves the morphological characteristics of human diploid fibroblasts as well.

EXAMPLE 8

Expression of IL-6 gene is not related to cellular senescence

Since fibroblasts are known to produce IL-6, the effect of basic-fibroblast growth factor and other cytokines on the expression of IL-6 was examined at different population doublings by bioassay and ELISA. $4x\ 10^4$ cells (2 ml) were incubated with and without cytokines (20 ng/ml) at 37°C for seven days and then cell supernatants were harvested for IL-6 determination. IL-6 levels in the human diploid fibroblasts were determined by bioassay as well as by ELISA (shown in parenthesis). All determinations were made in duplicate. As can be seen from TABLE I, as population doublings increase, there is an increased production of IL-6 which declines at later population doublings. In addition, tumor necrosis factor and Interleukin-1 induced large amounts of Interleukin-6 in these cells, whereas other cytokines including basic-fibroblast growth factor had no significant effects. Thus, it is clear that the extension of lifespan of cells by basic-fibroblast growth factor is not due to its effect on the expression of IL-6.

TABLE I

Effect of Cytokines on the Production of Interleukin-6 by HDF

Treatment	Interleukin-6 levels (ng/ml) Weeks in Culture			
	1	4	8	11
Control	6(8)	12(10)	24(14)	12(12)
bFGF	6(5)	12(14)	48(26)	6(6)
TNF	120(280)	480(440)	60(50)	0.3(0.3)
IL-1B	3840(3600)	3840(3200)	120(100)	0.3(0.5)
IL-4	6(14)	24(32)	12(14)	0.3(0.6)
IFN-a	3(8)	12(10)	6(10)	6(5)
IFN-B	3(8)	12(14)	6(6)	0.3(0)
IFN-g	6(8)	6(6)	24(12)	6(6)
TGF-b	12(14)	6(5)	NS	NS
LIF	6(6)	12(8)	6(7)	6(5)

EXAMPLE 9

Effect of bFGF on senescence-inducing genes

Human diploid fibroblasts at both young (population doubling of 23) and senescent (population doubling of 52) are treated with either basic fibroblast growth factor or served as controls. Subsequently, these cells are analyzed for expression by northern blot analysis of the genes which play a functional role in senescence. The northern blot analysis of mRNA is as follows: Total cellular RNA is extracted from cells according to procedures well known to one with ordinary skill in this art. For northern blot analysis, RNA samples (20 μg) are denatured with formaldehyde and formamide and electrophoresed in 0.8% agarose gel containing 0.67 M formaldehyde at 75 V for approximately 3 hours. RNA is alkali-transferred to Hybound N$^+$ nylon membranes (Amersham Corp., Arlington Heights, IL). After alkali transfer, the membranes are rinsed with 2X SSC (1X SSC; 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). The sample is prehybridized at 65°C for one hour in a buffer containing 7% SDS, 0.5 M sodium phosphate, 1 mM EDTA, pH 7.2 (hybridization buffer). The membranes are then hybridized for 16-20 hours with ^{32}P-labeled cDNA probes for various genes (approximate specific activity 5-10 x 10^8 cpm/ug DNA) in a hybridization buffer containing denatured salmon sperm DNA (0.2 mg/ml). After hybridization, membranes are washed twice with 2X SSPE (1 x SSPE: 0.18 M NaCl, 0.01 M sodium phosphate and 0.001 M EDTA) containing 0.1% SDS at room temperature, once with 1 X SSPE, 0.1% SDS at 65°C and finally with 0.1 X SSPE, 0.1% SDS at 65°C. The blots are then exposed to phosphoimager screen and the images are recorded and quantitated using "Image Quant" software. To demonstrate the equal loading of lanes, the probes were stripped off by washing the filters twice for 30 minutes each at 95°C with 0.5% SDS and rehybridized with labeled cDNA probes for glyceraldehyde 3-phosphate dehydrogenase GAPDH). A person having ordinary skill in this art would readily be able to obtain the necessary information concerning the sdi gene (See, e.g., Noda et al., Cloning of a senescent cell derived inhibitor of DNA synthesis using an expression scr n. Exp. Cell Res. 211: 90-98

Res. 211: 90-98 (1994) or the insulin growth factor-1 g ne (See, e.g., Ferber, et al., Failure of Senscent Human Fibroblasts to Express the Insulin-like Growth Factor-1 Gene. *J. Biol. Chem.* 268 : 17883-17888 (1993).

In the present invention, the effect of different cytokines on the replicative potential of human diploid fibroblasts was illustrated. The present invention demonstrated an increase in replicative potential, population doubling and total life span of human diploid fibroblasts by basic fibroblast growth factor, while a decrease in these parameters occurred with tumor necrosis factor, interferon-β, interferon-gamma, interleukin-1β and transforming growth factor-β; whereas interleukins-4 and -6, leukemia inhibitory factor and interferon-alpha had no effect. A continuous presence of cytokines was needed since short-term treatment of human diploid fibroblasts with cytokines provided results that had no relationship with long-term that obtained with human diploid fibroblasts in long term culture.

Lack of responsiveness of cells to certain cytokines could be in part due to the inability of senescent cells to express the IGF-1 gene, which could act as an autocrine as well paracrine growth factor. Basic fibroblast growth factor induction of the insulin growth factor -1 gene in human diploid fibroblasts may result in cellular proliferation.

The effect of different growth factors on the proliferation of fibroblasts have been examined, but only in short term culture. The present invention indicates that several cytokines independently enhance the proliferation of normal human diploid fibroblasts at an early stage in the *in vitro* lifespan but not when the cells become senescent.

Why senescent fibroblast fail to proliferate in response to growth factors is still not understood. Senescent fibroblasts are growth-arrested predominantly at the G1/S boundary of the cell cycle analogous to terminal differentiation. Several cell cycle-dependent genes (4F1, c-myc, JE-3, 2F1, ornithine decarboxylase, 2A9, p53, c-Ha-ras, β-actin, thymidylate kinase and histone H3) have been shown to be equally expressed

in young and senescent fibroblasts following serum stimulation. However other growth regulatory genes are repressed in senescent human diploid fibroblasts cells, including c-fos, cdc2, cyclin A and cyclin B. Senescent cells also underexpress the tissue inhibitor of metalloproteases and L7 ribosomal protein genes. Interestingly, several genes are overexpressed in senescent cells compared with young cells, including collagenase, α1-procollagen and cathepsin B. Recently, a gene termed the senescent derived inhibitor (*SDI*) was described and is overexpressed in senescent fibroblasts and is responsible for inhibition of DNA synthesis. The failure of senescent fibroblasts to enter S-phase has also been ascribed to its inability to phosphorylate retinoblastoma gene product. The shortening of telomeres, i.e., the terminal guanine rich sequence of chromosomes, are other characteristics of senescent human diploid fibroblasts. Whether any of these mechanisms are responsible for the lack of response of senescent cells to tumor necrosis factor is not clear.

Recently it was reported that tumor necrosis factor induces CDC2 and CDK2 gene expression in young, quiescent WI-38 fibroblasts but not in senescent fibroblasts. Since both of these protein kinases are essential to enable cells to enter into the S-phase; this may explain the lack of effect of tumor necrosis factor and other growth factors on the proliferation of senescent human diploid fibroblasts. Alternatively, senescent cells may also be unable to respond to tumor necrosis factor because of overexpression of *SDI* which has been shown to be a potent inhibitor of CDK2. However, this is unlikely because quiescent human diploid fibroblasts which also overexpress *SDI*, were found to be sensitive to proliferative effects of tumor necrosis factor. In contrast to senescent human diploid fibroblasts, tumor necrosis factor-induced proliferation of quiescent human diploid fibroblasts may be due to its ability to downmodulate *SDI* expression.

Treatment of different cell types, including human diploid fibroblasts by tumor necrosis factor causes the activation of a nuclear transcriptional factor NF-kB. The activation of NF-kB is an early event in TNF signal transduction

and is necessary for TNF-mediated induction of various cytokine genes including the interleukins, IL-2, IL-6, IL-8, TNF, lymphotoxin, interferon, GM-colony stimulating factor (GM-CSF), G-CSF, IL-2R, and human immunodeficiency virus-I (HIV-I) genes. The present invention demonstrates that the tumor necrosis factor-mediated activation of NF-kB occurs equally in young and senescent human diploid fibroblasts. Thus, the inability of senescent cells to proliferate or their reduced ability to produce interleukins in response to tumor necrosis factor appears to not be due to lack of activation of NF-kB.

Several reports have recently shown that there is age-related modulation of activation and production of cytokines from lymphocytes. The present invention demonstrates that there is a decline in the production of cytokines from senescent fibroblasts. Both the interleukins examined play an important role in inflammation.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Any patents and publications mentioned herein are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

Claims

1. A method of decreasing cellular senescence comprising the step of administering a pharmacologically effective dose of basic fibroblast growth factor to an animal.

2. The method of claim 1, wherein said animal is a human.

3. The method of claim 1, wherein said basic fibroblast growth factor is administered in a daily amount of from about 1 mg/kg to about 100 mg/kg.

4. A pharmaceutical composition for the inhibition of cellular senescence comprising a pharmacologically effective dose of basic fibroblast growth factor and a pharmaceutically acceptable carrier.

5. A method of increasing endogenous levels of IGF-1 in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal.

6. A method of decreasing endogenous levels of senescence inducing proteins in animals comprising the administration of a pharmacologically effective dose of basic fibroblast growth factor to an animal.

7. The method of claim 6, wherein said senescence inducing protein is sdi.

8. A method of treating a pathophysiological state characterized by undesirable fibroblast proliferation, comprising the administration of a pharmacologically effective dose of a fibroblast-inhibiting cytokine to an animal having said state.

9. The method of claim 8, wherein said pathophysiological state is sel cted from the group sel cted from

psoriasis, arthritis, restenosis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, scleroderma and hemangioma.

10. The method of claim 8, wherein said animal is a human.

11. The method of claim 8, wherein said fibroblast-inhibiting cytokine is selected from the group consisting of tumor necrosis factor, transforming growth factor-β, interferon-β, interferon-gamma and interleukin-1β.

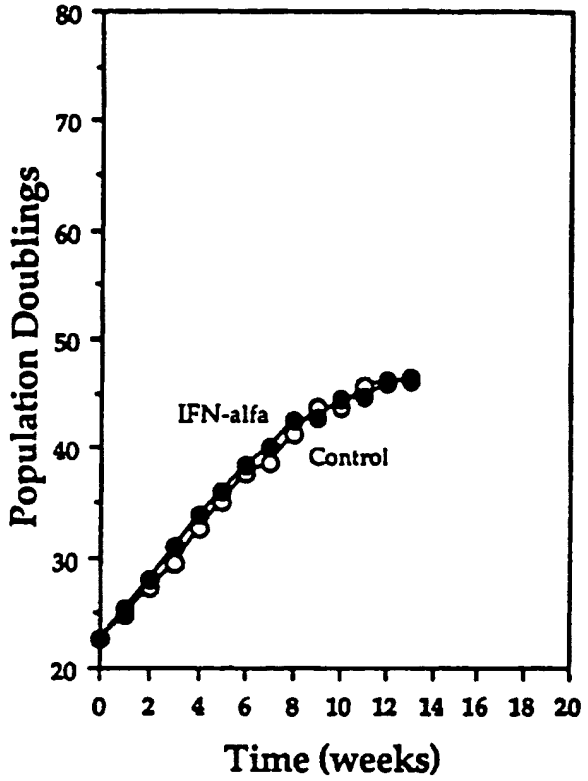


FIG. 1

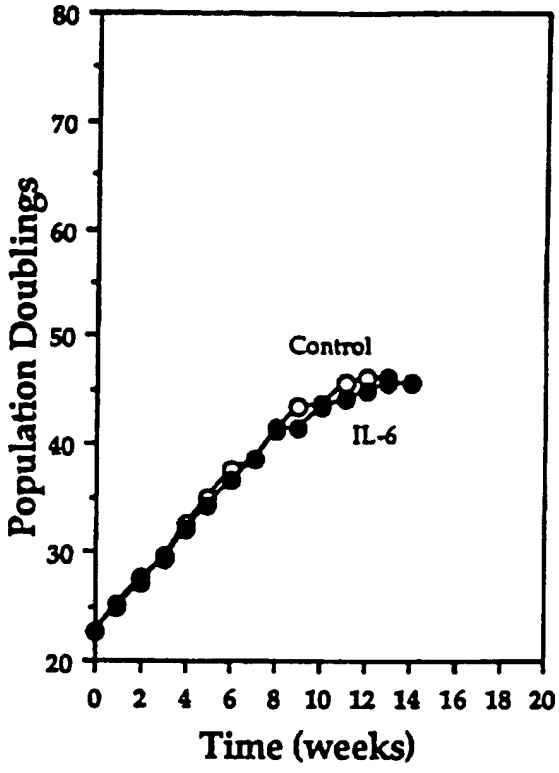


FIG. 2

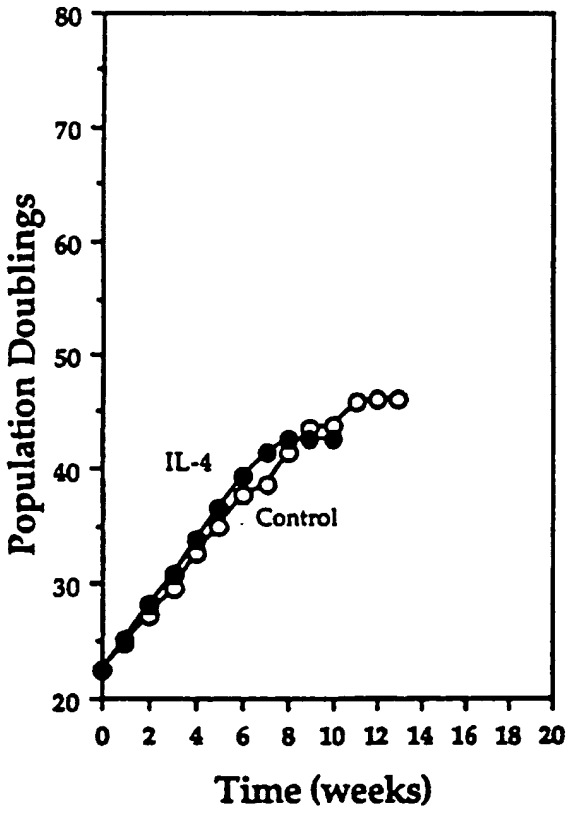


FIG. 3

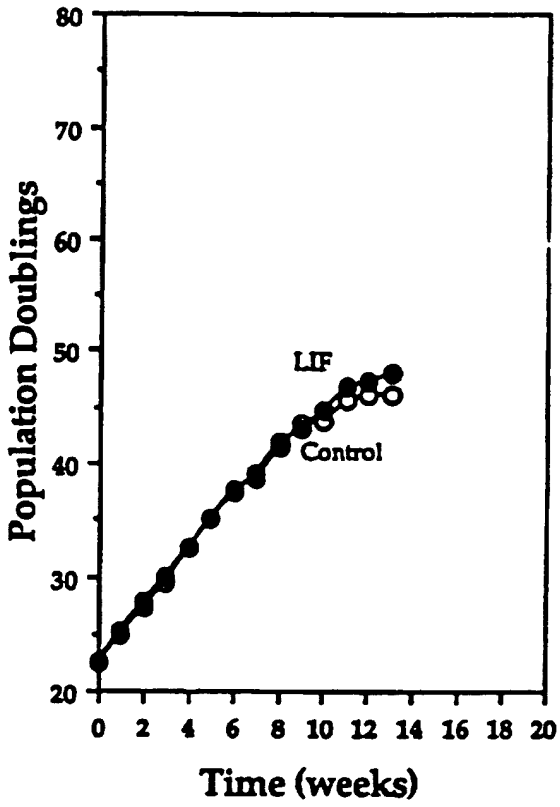


FIG. 4

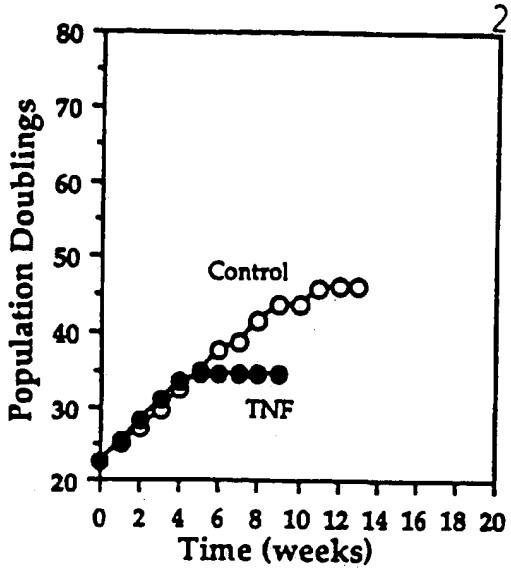


FIG. 5

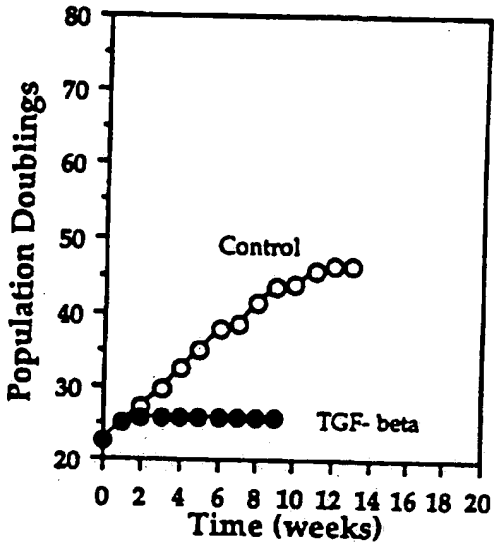


FIG. 6

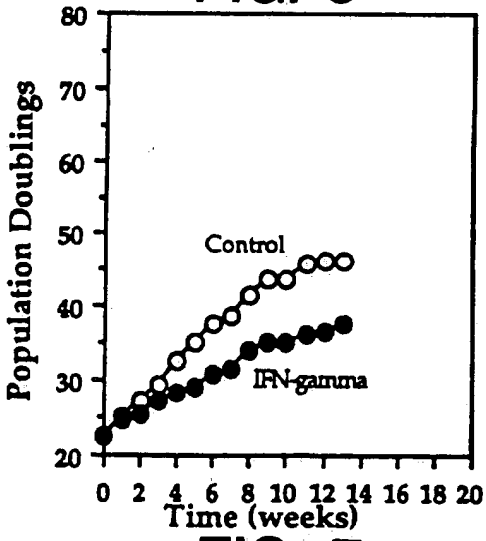


FIG. 7

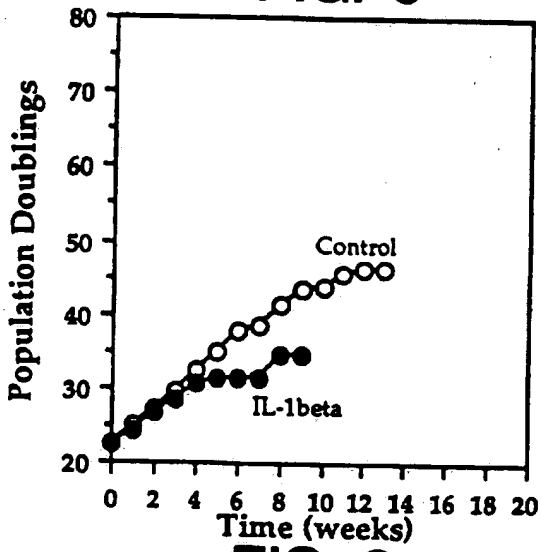


FIG. 8

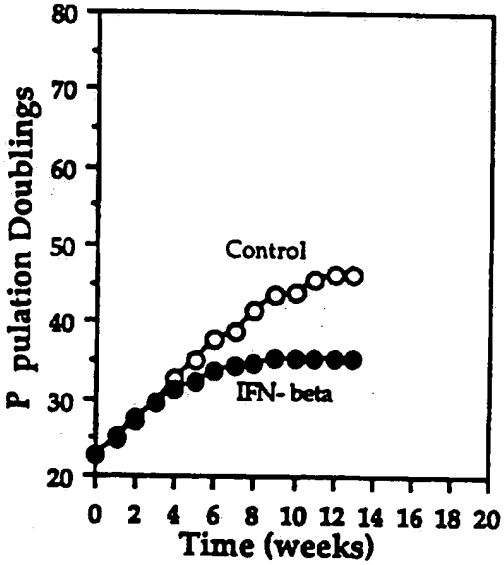


FIG. 9

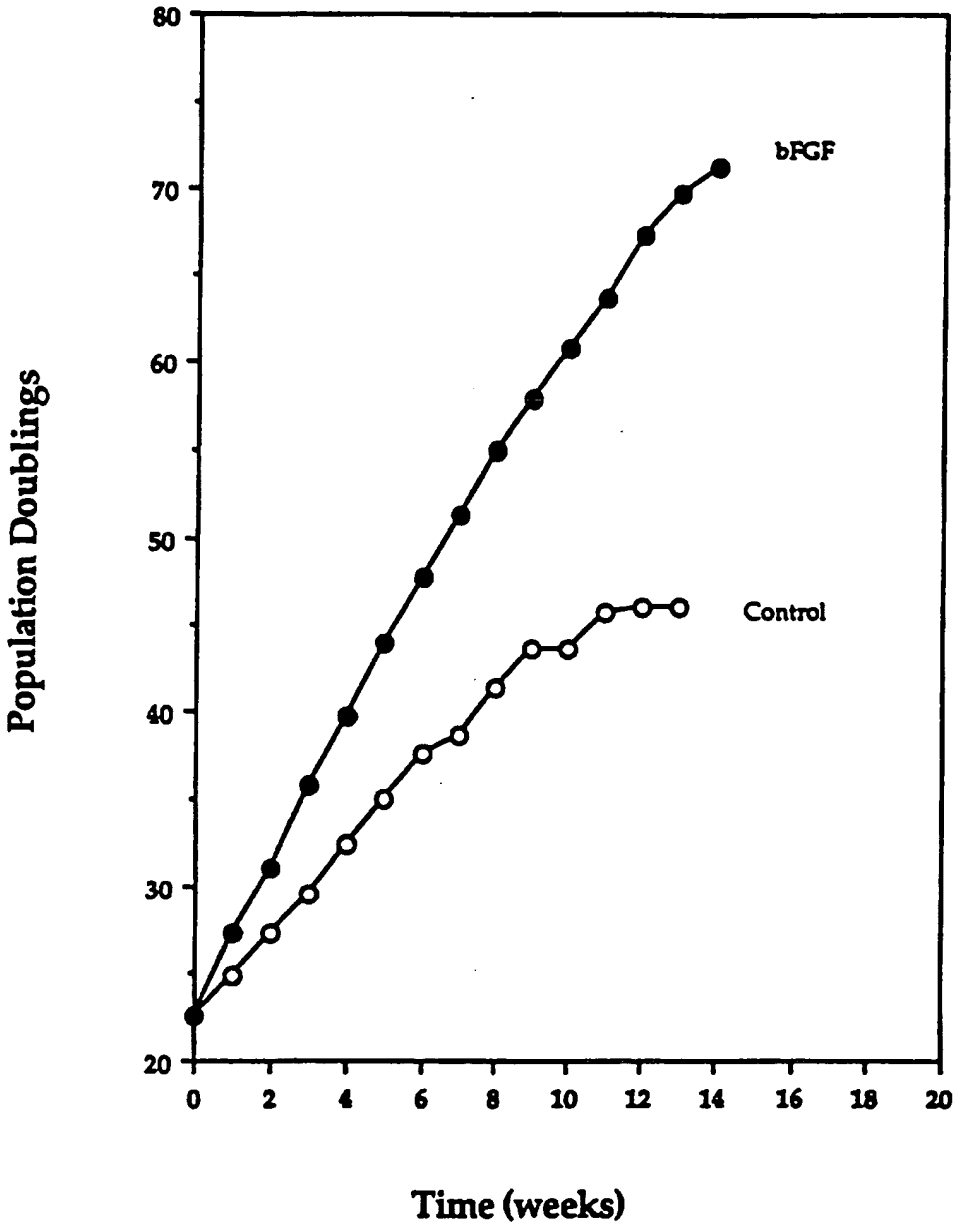
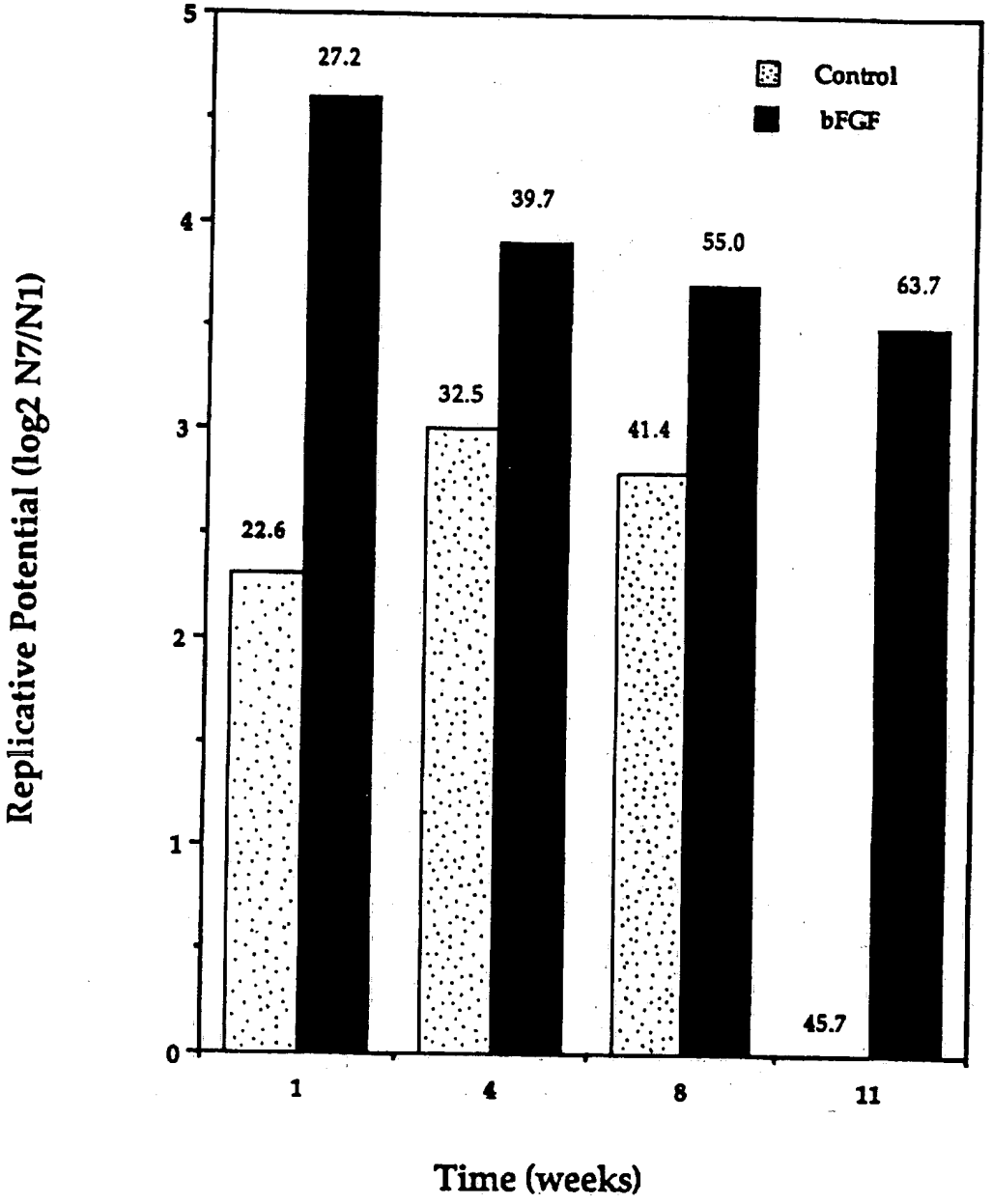

80
70
60
50
40
30
20
0
2
4
6
8
10
12
14
16
18
20
bFGF
Control
Population Doublings
Time (weeks)

FIG. 10



Numbers on the bars indicate cumulative population doublings

FIG. 11

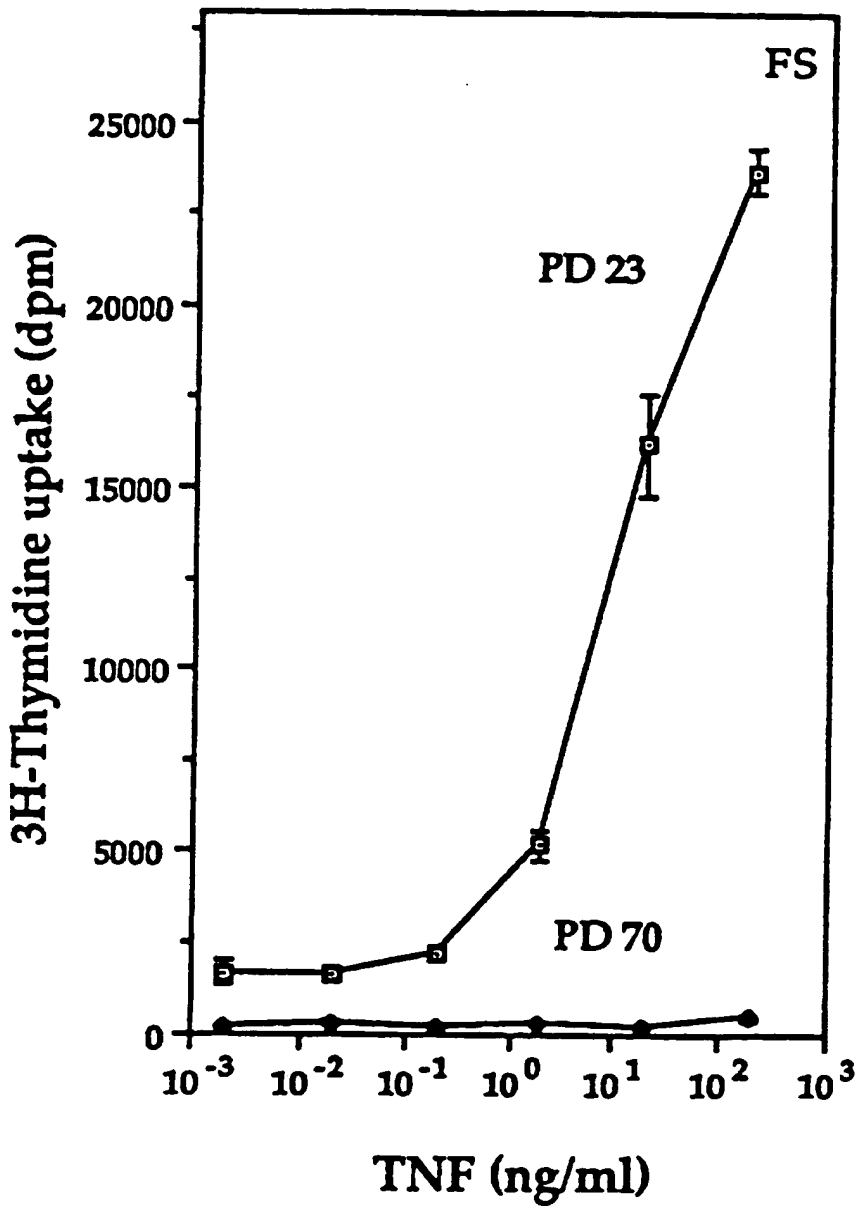

FS
PD 23
PD 70
25000
20000
15000
10000
5000
0
3H-Thymidine uptake (dpm)
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
TNF (ng/ml)

FIG. 12A

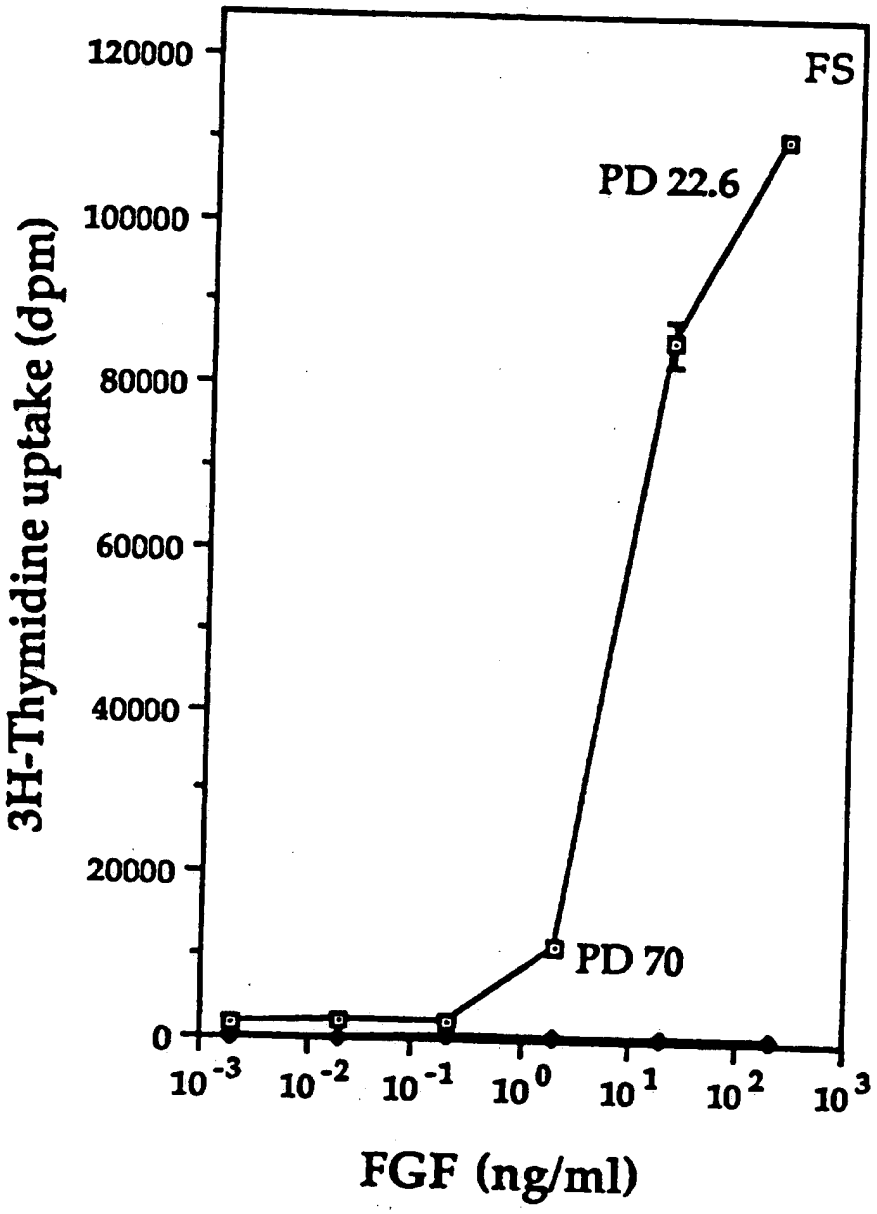

120000
100000
80000
60000
40000
20000
0
3H-Thymidine uptake (dpm)
FS
PD 22.6
PD 70
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
FGF (ng/ml)

FIG. 12B

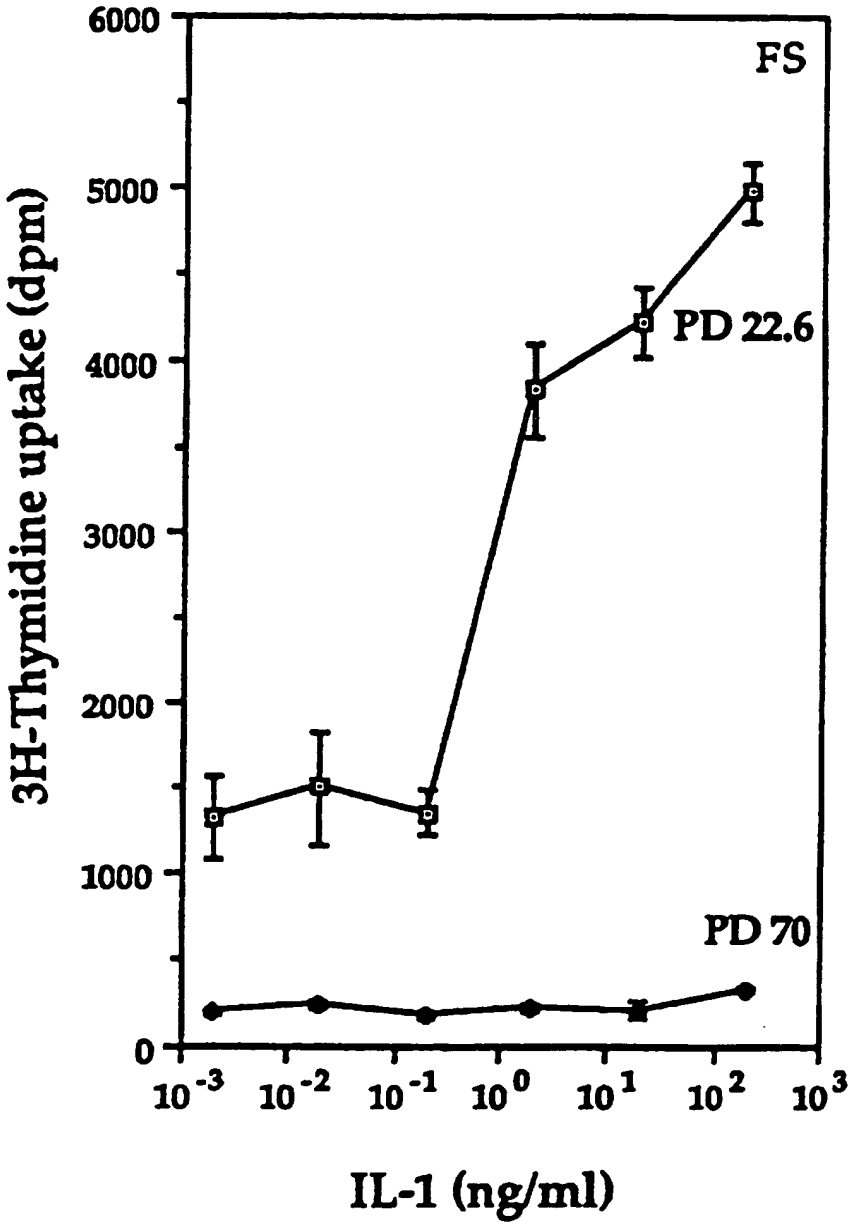
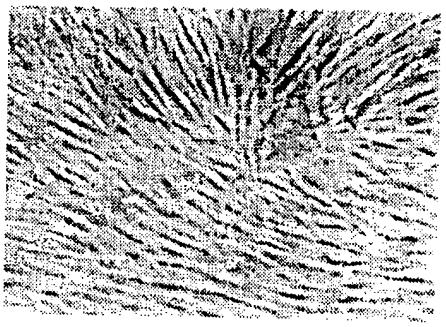
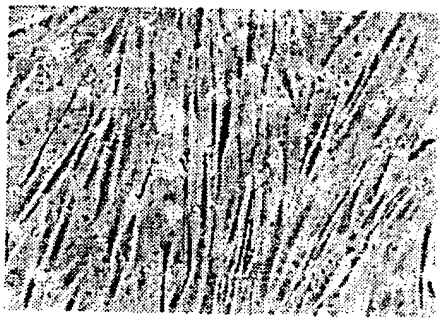

6000
5000
4000
3000
2000
1000
0
3H-Thymidine uptake (dpm)
FS
PD 22.6
PD 70
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
IL-1 (ng/ml)

FIG. 12C

PD 33.8 (4 wks)



PD 46.4 (13 wks)



PD 46.4 (14 wks)

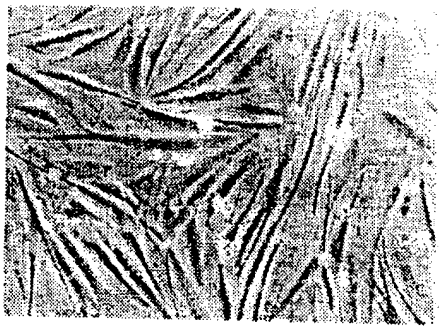
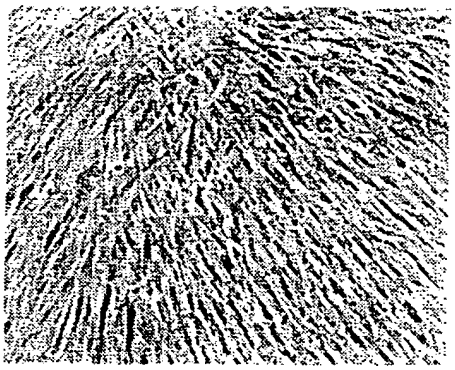
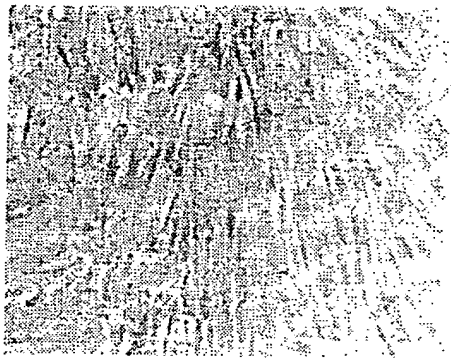


FIG. 13

PD 33.8 (4 wks)



PD 42.5 (8 wks)



PD 42.5 (13 wks)

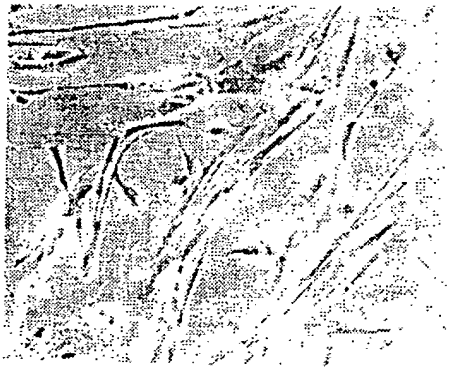


FIG. 14

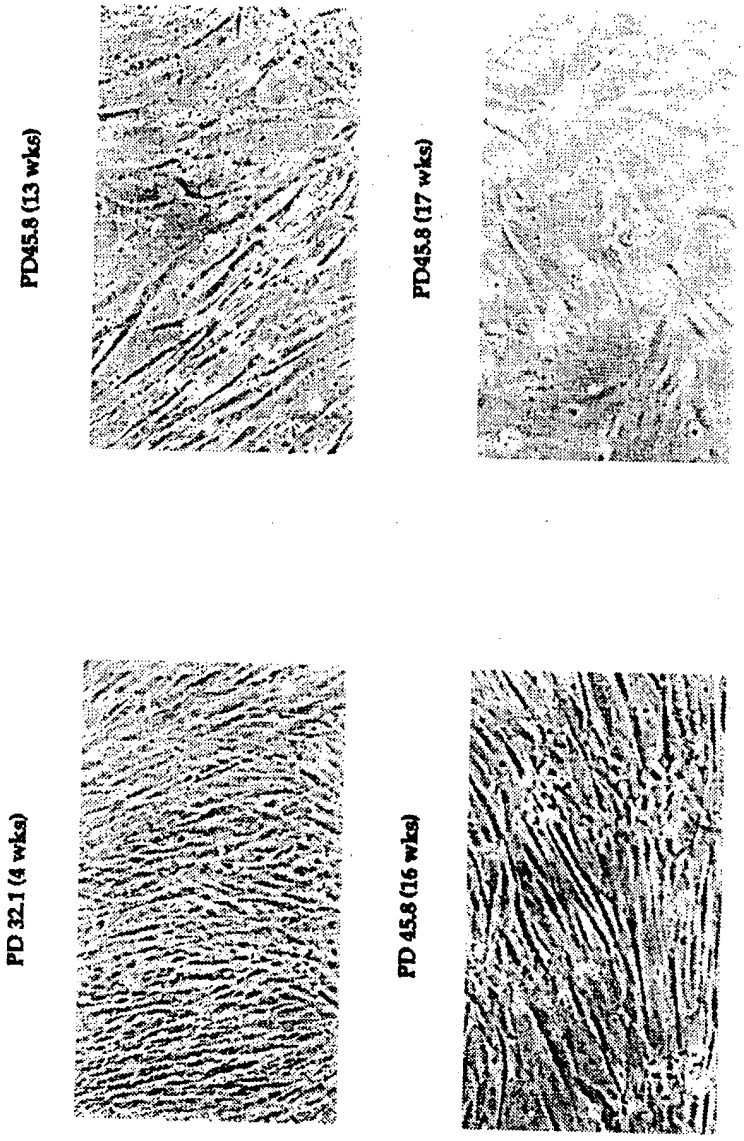
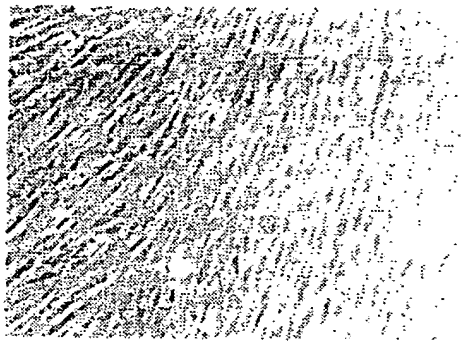
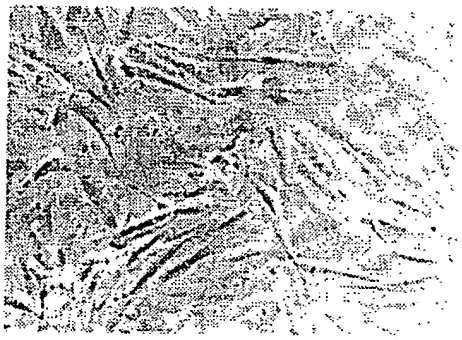


FIG. 15

PD 32.5 (4 wks)



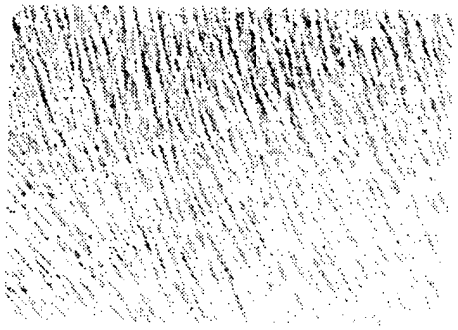
PD 47.9 (13 wks)



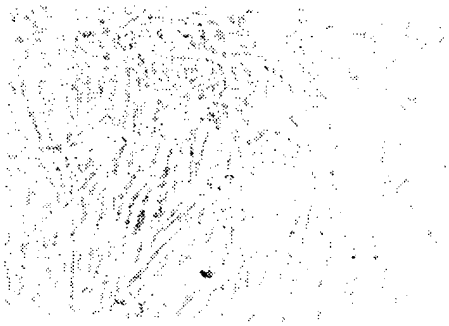
PD 47.9 (14 wks)

FIG. 16

PD 30.5 (4 wks)



PD 34.4 (8 wks)



PD 34.4 (9 wks)

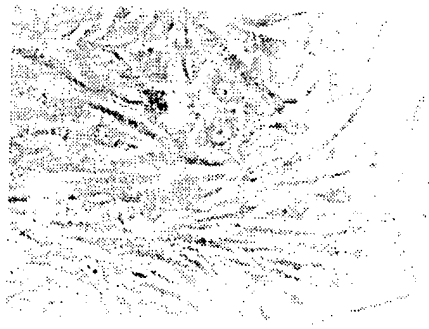


FIG. 17

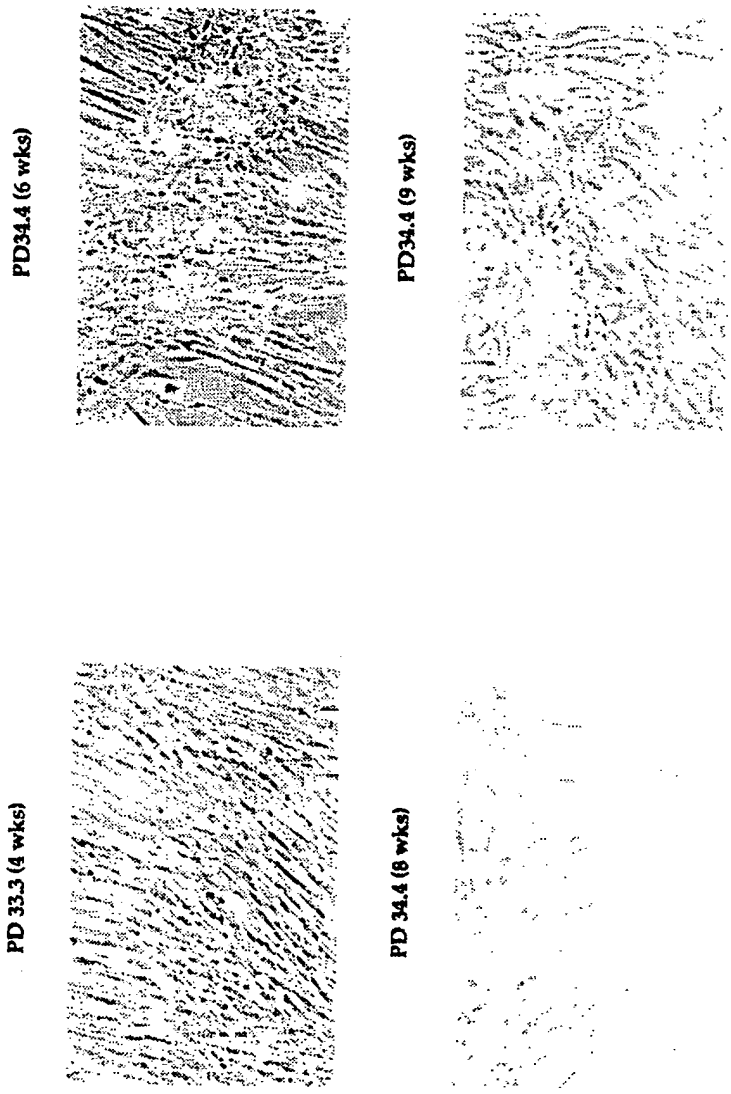
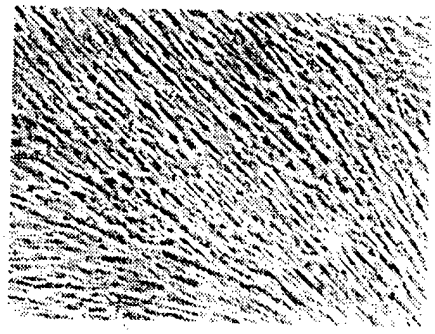


FIG. 18

PD 28.4 (4 wks)

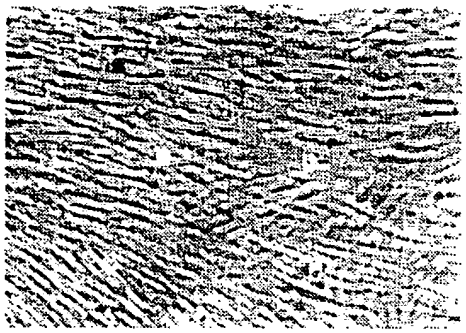


PD 37.5 (13 wks)

PD 37.5 (14 wks)

FIG. 19

PD 31.1 (4 wks)

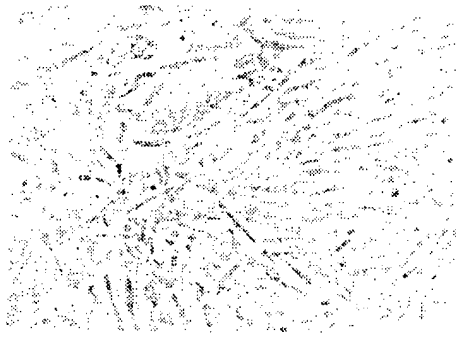


PD 35.3 (13 wks)

PD 35.3 (14 wks)

FIG. 20

PD 25.6 (4 wks)



PD 25.6 (6 wks)

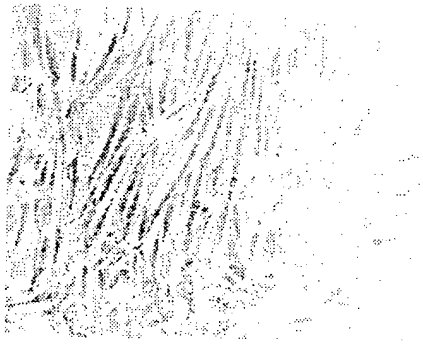


FIG. 21

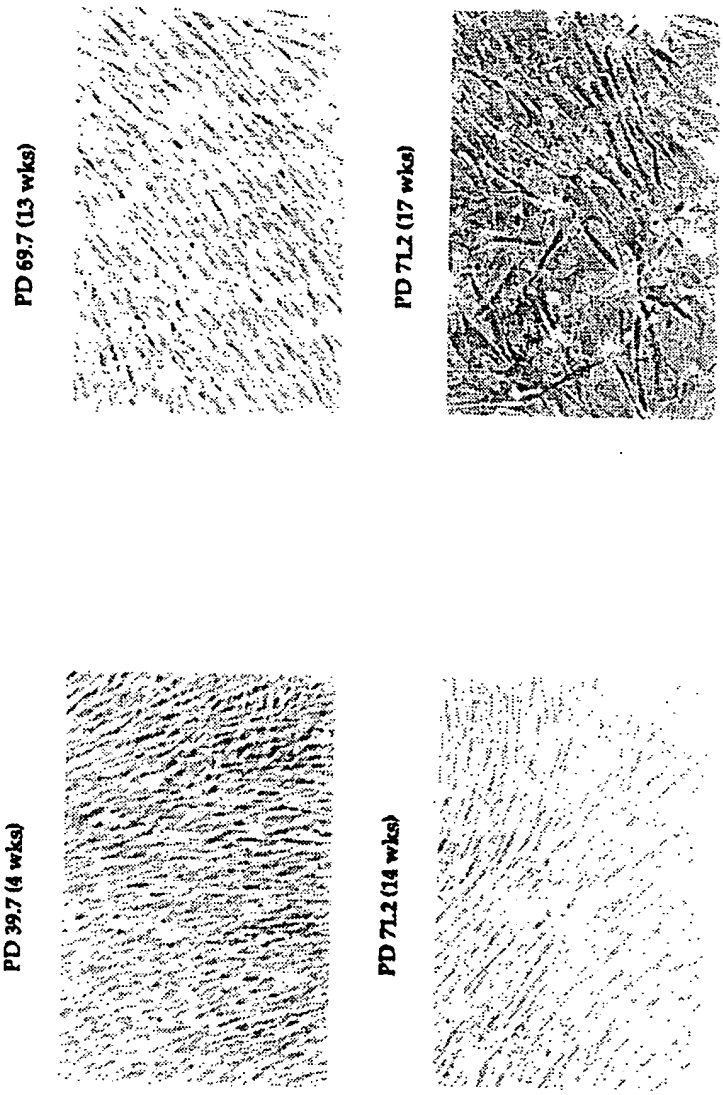
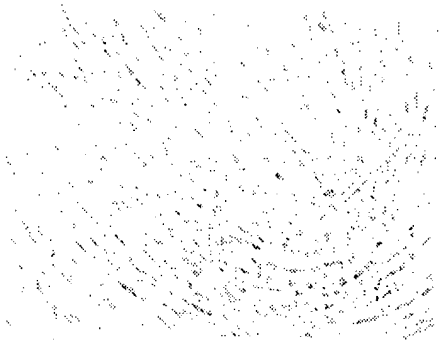


FIG. 22

PD 32.5 (4 wks)



PD 46.1 (13 wks)

PD 46.1 (14 wks)

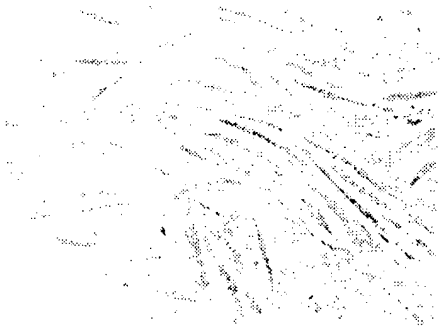


FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10457

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00
US CL : 514/21, 824, 825, 863

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/21, 824, 825, 863

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 111, issued 1989, Froger-Gaillard Et Al., "Growth-Promiting Effects Of Acidic And Basic Fibroblast Growth Factor On Rabbit Articular Chondrocytes Aging In Culture", Abstract No. 67706, Exp. Cell Res., 183(2), pages 388-98, see entire Abstract.	1-4
Y	Chemical Abstracts, Volume 114, issued 1991, Wilson Et Al., "Basic Fibroblast Growth Factor Stimulates Myelopoiesis In Long-Term Human Bone Marrow Cultures", Abstract No. 178582, Blood, 77(5), pages 954-60, see entire abstract.	1-4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 NOVEMBER 1995	14 DEC 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KIMBERLY JORDAN Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10457

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Abstracts, Volume 116, issued 1991, Wilson Et Al., "Stimulation of Bone Marrow Stromal and Progenitor Cells With a Fibroblast Growth Factor", abstract no. 100165, WO 9118620 A1, see entire abstract.	1-4
Y	Chemical Abstracts, Volume 119, issued 1993, Mishima Et Al., "Cosmetic Skin Preparations Containing Basic Fibroblast Growth Factor (bFGF)", abstract no. 15115, JP 05043442 A2, see entire abstract.	1-4
Y	Chemical Abstracts, Volume 118, issued 1992, Penhoat Et Al., "Cultured Adrenal Cells Are The Site of Action and Secretion of Insulin-Like Growth Factor I (IGF-I)", abstract no. 226448, Colloq. Inserm, 222(Cell. Mol. Biol. Adrenal Cortex), pages 273-81, see entire abstract.	5
Y	Chemical Abstracts, Volume 110, issued 1989, Duncan Et Al., "Differential Regulation of Glycosaminoglycan, Fibronectin, and Collagenase Production in Cultured Human Dermal Fibroblasts by Interferon-Alpha, -Beta, and -Gamma", abstract no. 190774, Arch. Dermatol. Res., 281(1), pages 11-18, see entire abstract.	8-11
Y	Chemical Abstracts, Volume 110, issued 1988, Bryckaert Et Al., "Transforming Growth Factor (TGF.BETA) Decreases the Proliferation of Human Bone Marrow Fibroblasts by Inhibiting the Platelet-Derived Growth Factor (PDGF) Binding", abstract no. 34366, Exp., Cell Res., 179(2), pages 311-21, see entire abstract.	8-11
Y	US, A, 5,312,621 (BERMAN ET AL.) 17 May 1994, see Abstract and Column 2, Line 44 - Column 3, Line 6.	8-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10457

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10457

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS and CAS ONLINE: basic fibroblast growth factor, tumor necrosis factor, transforming growth factor?, interferon?, interleukin? with insulin growth factor, senescence, mortalin, sdi, senescence inducing protein, fibroblast?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, drawn to a method and composition of decreasing cellular senescence by administering a basic fibroblast growth factor.
Group II, claim(s) 5, drawn to a method of increasing endogenous levels of senescence by administering a basic fibroblast growth factor.
Group III, claim(s) 6-7, drawn to a method of decreasing endogenous levels of senescence inducing proteins by administering a basic fibroblast growth factor.
Group IV, claim(s) 8-11, drawn to a method of treating pathophysiological state characterized by undesirable fibroblast proliferation by administering a fibroblast-inhibiting cytokine.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The methods of groups I-IV are drawn to different methods of increasing and decreasing various physiological conditions each of which have no clear nexus with the other conditions. Thus, each method has its own separate special technical feature.